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ORIGINAL ARTICLE

Antibodies against ARHGDIB are associated with long-term kidney graft loss

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Abbreviations: ABMR, Antibody-mediated rejection; AKME, Adjusted Kaplan-Meier estimator; APMA donor-specific P, Adipocyte plasma membrane-associated protein; ARHGDIB, Rho GDP-dissociation inhibitor 2; ARHGEF6, Rho guanine nucleotide exchange factor 6; AT1R, Angiotensin II type 1 receptor; CDC-XM, Complement-dependent cytotoxicity crossmatch; CI, Confidence interval; CIT, Cold ischemia time; DBD, Donation after brain death; DCD, Donation after cardiac death; DSA, Donor-specific anti-HLA antibodies; ETAR, Endothelin type A receptor; HLA, Human leukocyte antigen; HR, Hazard ratio; IL, Interleukin; IPW, Inverse probability weighting; LPLUNC1, BPI fold-containing family B member 1; MFI, Median fluorescence intensity; MMF, Mycophenolate mofetil; NOTR, Netherlands Organ Transplant Registry; PEGR, Peroxisomal trans-2-enoyl-CoA reductase; PLA2R, Phospholipase A2 receptor; PRA, Panel reactive antibodies; PRKZ, Protein kinase C zeta type; STBRs, Signal-to-background ratios; Tub4B, Tubulin beta-4B.

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The clinical significance of non-HLA antibodies on renal allograft survival is a matter of debate, due to differences in reported results and lack of large-scale studies incorporating analysis of multiple non-HLA antibodies simultaneously. We developed a multiplex non-HLA antibody assay against 14 proteins highly expressed in the kidney. In this study, the presence of pretransplant non-HLA antibodies was correlated to renal allograft survival in a nationwide cohort of 4770 recipients transplanted between 1995 and 2006. Autoantibodies against Rho GDP-dissociation inhibitor 2 (ARHGDIB) were significantly associated with graft loss in recipients transplanted with a deceased-donor kidney (N = 3276) but not in recipients of a living-donor kidney (N = 1496). At 10 years after deceased-donor transplantation, recipients with anti-ARHGDIB antibodies (94/3276 = 2.9%) had a 13% lower death-censored covariate-adjusted graft survival compared to the anti-ARHGDIB-negative (3182/3276 = 97.1%) population (hazard ratio 1.82; 95% confidence interval, 1.32-2.53; $P = .0003$). These antibodies occur independently from donor-specific anti-HLA antibodies (DSA) or other non-HLA antibodies investigated. No significant relations with graft loss were found for the other 13 non-HLA antibodies. We suggest that pretransplant risk assessment can be improved by measuring anti-ARHGDIB antibodies in all patients awaiting deceased-donor transplantation.

KEYWORDS

ARHGDIB, kidney transplantation, non-HLA antibodies

1 | INTRODUCTION

Chronic kidney disease affects about 10% of the global population.¹ Over 2 million people worldwide currently receive dialysis treatment, or are recipients of a kidney transplant. Kidney transplantation is the preferred treatment for end-stage renal disease due to superior quality of life and survival rates. However, although short-term renal allograft survival has improved considerably in the last 20 years, antibody-mediated rejection (ABMR) remains one of the major causes of graft loss and of deterioration of graft function in the long-term. Donor-specific anti-HLA antibodies (DSA) are well known to play an important role in this process. Nonetheless, (subclinical) ABMR occurs also in the absence of DSA, which has sparked interest in the short- and long-term clinical relevance of donor-reactive antibodies recognizing proteins other than HLA (ie, non-HLA-antibodies).²

The clinical relevance of non-HLA antibodies on graft survival is not clear.^{3,4} Although it has been reported that non-HLA antibodies against Angiotensin II type 1 receptor (AT1R) are an independent risk factor for long-term graft loss,⁵ others could not replicate these findings,^{6,7} which may be caused by the inclusion or exclusion of patients with pretransplant DSA. However, antibody-mediated rejection of renal allografts from HLA-identical sibling donors and also from deceased donors in

the absence of pretransplant DSA has been reported several times.^{8,9} And recently it was reported that the development of circulating natural antibodies posttransplant is associated with poorer graft survival, worse graft function, and more microvascular injury.¹⁰

Non-HLA antibodies have been described against a variety of targets, but large-scale studies incorporating analysis of these antibodies simultaneously to assess their clinical relevance in kidney transplantation are lacking. In this retrospective study on a large national cohort of 4770 renal transplant recipients, we assessed the impact on graft survival of IgG autoantibodies against 14 previously identified target proteins.¹¹

2 | MATERIALS AND METHODS

2.1 | Study population

Between January 1995 and December 2005, 6097 kidney transplantations with a negative complement-dependent cytotoxicity crossmatch were performed in The Netherlands. Clinical data were obtained from the Dutch Organ Transplant Registry. The use of sera and experimental protocols was approved by the Research Ethics Committee for Biobanks and the Medical Ethics Committee of the

University Medical Center Utrecht. Experimental protocols were performed in accordance with the Federation of Dutch Medical Scientific Societies Code of Conduct. The study was conducted in accordance with the 2013 Declaration of Helsinki and the 2008 Declaration of Istanbul. Of 4787/6097 (78%) transplantations, pretransplant serum was available. Seventeen transplantations were excluded due to lack of follow-up, and the remaining 4770 transplantations were included in this analysis. Minimal follow-up time was 10 years after transplantation. Data available on request due to privacy/ethical restrictions.

2.2 | Assessment of non-HLA antibodies

We selected 14 non-HLA target proteins from the literature.¹¹ Antibodies against the glomerular basement membrane protein agrin have been described in the context of transplant glomerulopathy.¹² Antibodies against adipocyte plasma membrane-associated protein (APMAP), Rho GDP-dissociation inhibitor 2 (ARHGDIB), Rho guanine nucleotide exchange factor 6 (ARHGEF6), Lamin B1, BPI fold-containing family B member 1 (LPLUNC1), protein kinase C zeta type (PRKCZ), and tubulin beta-4B (Tubb4B) were all demonstrated either in chronic hemodialysis patients or patients awaiting kidney transplantation. Antibodies directed against targets expressed on the endothelium, that is, AT1R and endothelin type A receptor (ETAR), were reported to be involved directly or indirectly in renal disease. Antibodies against the intracellular proteins vimentin and peroxisomal trans-2-enoyl-CoA reductase (PECR) have been reported to be associated with allograft failure.^{13,14} Pre- and posttransplant endorepellin antibody levels were increased in patients with vascular rejection¹⁵ and phospholipase A2 receptor (PLA2R) antibodies are strongly associated with primary membranous nephropathy.¹⁶ We commercially purchased all proteins with the exception of PLA2R, which was produced in-house, and coupled these proteins directly with carboxylated MagPlex Microspheres (Luminex Corp, Austin, TX) as recommended by Luminex.¹⁷ In addition, we produced 12 of the 14 proteins with a HaloTag, with the exception of AT1R and PECR. The HaloTag proteins were coupled via a HaloTag Amine (O4) Ligand (Promega, Madison, WI) to the carboxylated MagPlex microspheres. A detailed description of the proteins and the coupling methods is given elsewhere.¹¹

A mix of 31 different microspheres was made containing 15 directly coupled proteins (transferrin and 14 target proteins), 13 in-house produced HaloTag-coupled proteins (transferrin and 12 target proteins), an IgG-coupled microsphere as a positive control, and a HaloTag amine-coupled and empty microsphere as additional negative controls. Transferrin (directly- or HaloTag-coupled) also served as a negative control, since it is ubiquitously present and no autoantibodies against transferrin have been reported. Sera (1:25 dilution) were incubated overnight with the microsphere mix. Next, R-phycoerythrin-conjugated goat-anti human antibody was added. After 30 minutes of incubation, wash buffer was added and samples were measured on a Luminex 200 flow analyzer (Luminex Corp).

2.3 | Histology

Three-micrometer formalin-fixed paraffin-embedded sections were used. After antigen retrieval in citrate solution pH 6, primary antibody against ARHGDIB (Biobyte, San Francisco, CA) was applied in a 1:2000 solution for 1 hour at room temperature, followed by HRP-labeled polymer anti-rabbit Ig detection antibody (BrightVision, VWR, Duiven). Bound antibody was visualized with Nova Red substrate (Vector labs), and finally nuclei were counterstained with hematoxylin.

2.4 | Statistical analysis

Because the Kaplan-Meier estimates were biased due to unbalanced distribution of confounders, death-censored graft survival was assessed using the adjusted Kaplan-Meier estimator (AKME) based on inverse probability weighting (IPW). Each observation is weighted by its inverse probability of being in a certain group.¹⁸ Hazard ratios (HRs) and 95% confidence intervals (95% CIs) were derived using multivariable Cox regression. A Bonferroni correction was used to adjust for multiple comparisons when studying the effects of non-HLA antibodies on graft survival and $P < .002$ (.05 divided by 25 non-HLA antibodies) was considered as statistically significant (applied for analyses in Tables 1, S3, and S4). We adjusted in both the AKME and Cox regression for recipient age (quadratic) and donor age (quadratic), cold ischemia time (for donation after brain death or cardiac death), years on dialysis (quadratic), induction therapy

TABLE 1 Multivariable analyses of the effect of antibodies against ARHGDIB on 10-year death-censored graft failure

	No. (%) of transplantations with anti-ARHGDIB antibodies	Hazard ratio	95% CI	P-value
Total cohort (N = 4770)	134 (2.8)	1.701	1.265-2.288	.0004
Deceased donors (N = 3276)	94 (2.9)	1.820	1.318-2.531	.0003
Living donors (N = 1494)	40 (2.7)	1.249	0.587-2.657	.5639

Note: In this multivariable analysis we evaluated the effect of the presence of pretransplant ARHGDIB on the 10-year death-censored graft failure and adjusted for differences in the following covariates: recipient age (quadratic), donor age (quadratic), donor type (living or deceased, only for the total cohort), cold ischemia time in hours for donation after brain death (DBD) and donation after cardiac death (DCD), time on dialysis in years (quadratic), induction therapy with Interleukin-2 receptor-blocking antibody and the presence of pretransplant donor-specific anti-HLA antibodies against HLA-A/B/DR/DQ. CI, confidence interval. A Bonferroni correction was used to adjust for multiple comparisons with $P < .002$ considered as statistically significant.

with IL-2 receptor blocker, and the presence of pretransplant single antigen bead-defined DSA against HLA-A/B/DR/DQ. (For more detailed descriptions see Kamburova et al.¹⁹) Statistical analyses were performed with R version 3.4.1 and SAS version 9.4 (SAS Institute, Cary, NC). Continuous data were analyzed with the Mann-Whitney *U* test and categorical data with the chi-square test.

3 | RESULTS

3.1 | Determination of a clinically relevant cut-off for the presence of non-HLA antibodies

We analyzed the 4770 pretransplant sera using our multiplex non-HLA assay, and the individual median fluorescence intensity (MFI) values with box and whisker plots are shown in Figure S1. As we observed relatively high background signals for some sera (Figure S1A), we decided to use signal-to-background ratios (STBRs) as a parameter to determine non-HLA antibody positivity. The correlation between the MFI of transferrin (directly- or HaloTag-coupled) and the MFIs of target microspheres was stronger than the correlation between MFI of other negative control microspheres and that of target microspheres (data not shown). Therefore, transferrin was selected as the most optimal negative control, and was used correspondingly to calculate the STBRs (Figure S2). To determine the clinically relevant cut-off, we analyzed the impact on 1-, 5-, and 10-year death-censored graft survival of various cut-offs for each antibody in a univariate analysis (Figure 1). For each non-HLA antibody, we selected the ratio and absolute MFI cut-off that resulted in the highest difference in graft survival between the antibody-negative and antibody-positive group. For ARHGDIB, a ratio of 10 in combination with an absolute MFI of 500 was chosen as cut-off values using the directly coupled microspheres, resulting in 134/4770 (2.8%) positive patients (Figure 1). An overview of the selected cut-offs for the other non-HLA antibodies based on the maximal graft survival difference is summarized in Table S1. Depending on the type of non-HLA antibody analyzed, percentages of positive sera ranged from 0.9% to 2.8%, and varying differences were observed in 1-, 5-, and 10-year graft survival.

3.2 | Impact of pretransplant non-HLA antibodies on long-term graft survival

After Bonferroni correction, a significant difference in graft survival was observed between patients with pretransplant antibodies against ARHGDIB compared to patients without antibodies against this target (Table 1). No significant relation with graft loss was observed for the other non-HLA antibodies. A summary of the impact of the other non-HLA antibodies on 10-year graft survival of the total cohort is summarized in Table S2.

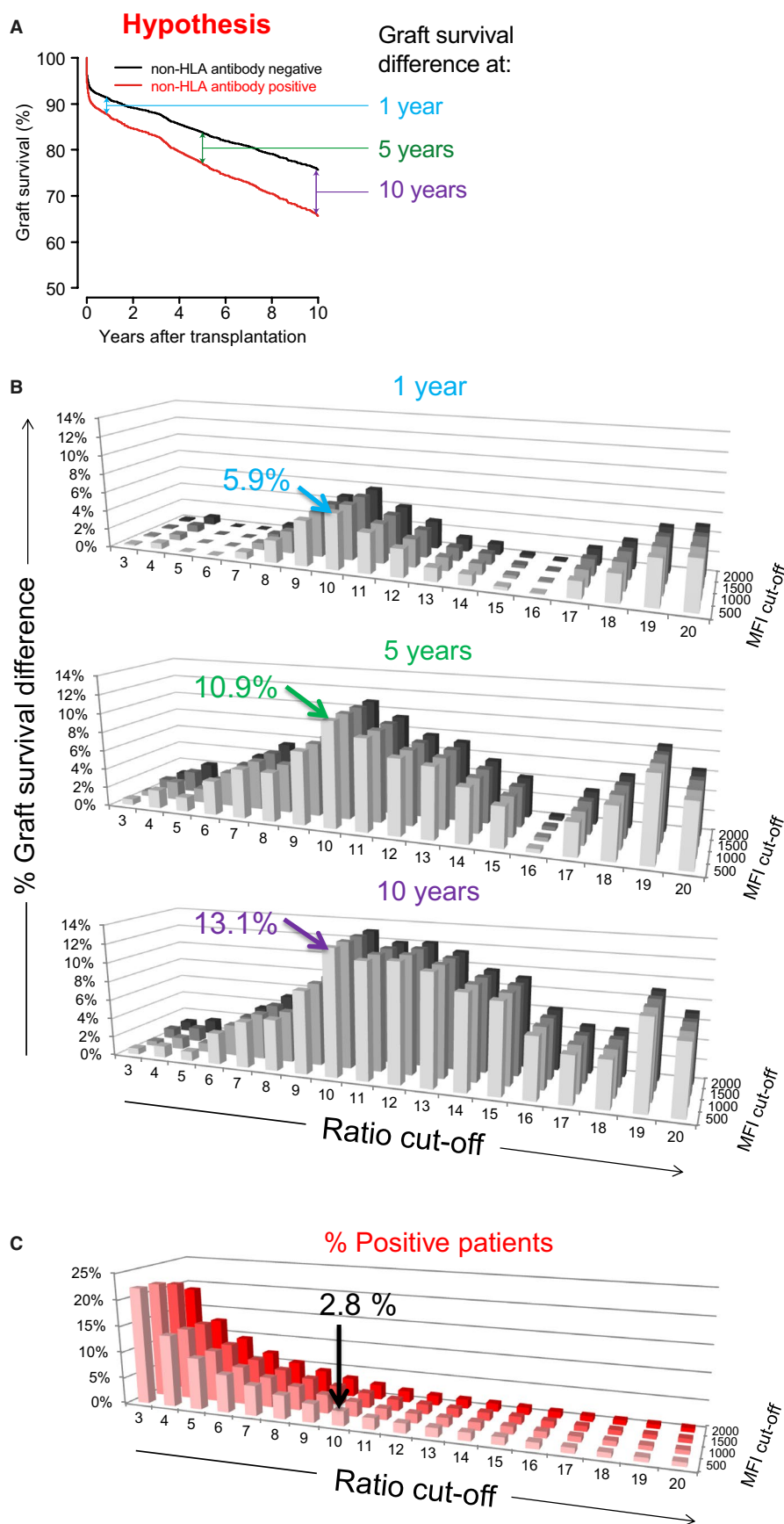
Because our cohort contained a relatively high proportion of living donors and we previously found that DSA had mainly an impact on deceased-donor transplantations with only a limited effect on living-donor transplantations,¹⁹ we also analyzed the impact of non-HLA antibodies on long-term graft survival according

to donor status (3276 deceased-donor and 1494 living-donor transplantations). After deceased-donor transplantation, the AKME according to the presence of ARHGDIB antibodies showed a 10-year death-censored graft survival of 61% (95% CI 50%-70%) for the 94 of 3276 patients with and 73% (95% CI 71%-75%) for the 3182/3276 patients without ARHGDIB antibodies (Figure 2A; $P = .017$). Pretransplant DSA against HLA-A/B/DR/DQ was found in only 10 of 94 patients (10.6%) with ARHGDIB antibodies and 423/3182 patients (13.3%) without ($P = .454$). A Kaplan-Meier analysis in this small subgroup did not show an indication for a synergistic effect of DSA and anti-ARHGDIB antibodies. In addition, we could find no indication for interaction between the presence of all studied autoantibodies, or an effect of their combined presence on graft survival. There were no significant differences between the patient, donor, and transplant characteristics between the anti-ARHGDIB positive and negative groups transplanted with a deceased-donor kidney, except the cold ischemia time was slightly longer in anti-ARHGDIB-positive patients (23.5 ± 7.7 hours) compared to anti-ARHGDIB-negative patients (21.8 ± 7.2) transplanted with a deceased donor ($P = .043$, Table 2). The multivariable analysis, adjusted for the same covariates, showed that the presence of ARHGDIB antibodies was associated with a higher risk of 10-year graft failure after transplantation with a deceased-donor kidney (Table 1, HR 1.82, 95% CI 1.32-2.53, $P = .0003$). At 1 year after transplantation, the HR was 1.620 (95% CI, 0.993-2.643) for the anti-ARHGDIB-positive group compared to the anti-ARHGDIB-negative group (Table S3). Furthermore, the rejection-free survival was comparable for patients with and without antibodies against ARHGDIB (Figure S3). For the living-donor transplantations, the presence of ARHGDIB antibodies was not associated with decreased graft survival (Figure 2B) or increased risk of graft failure in multivariable analysis (Table 1, HR 1.25, 95% CI 0.59-2.66, $P = .56$). Finally, we also assessed the association between type of donor, anti-ARHGDIB antibodies, and 10-year graft survival in the same model. The hazard ratio for a living donor was 0.563 (95% CI 0.488-0.650, $P < .0001$) compared to deceased donors, suggesting that anti-ARHGDIB was significantly associated with graft loss in recipients transplanted with a kidney from a deceased donor but not in recipients of a living-donor kidney.

3.3 | ARHGDIB expression in the kidney

Finally, we were wondering where in the kidney ARHGDIB is expressed. Therefore, we stained biopsies of a transplanted kidney without histological abnormalities and a transplanted kidney with acute tubular necrosis using an anti-ARHGDIB antibody. In a transplanted kidney without histological abnormalities, weak ARHGDIB expression was seen in endothelial cells of interlobular arteries, endothelial cells of peritubular capillaries, and endothelial cells of glomerular capillaries (Figure 3A). In a transplanted kidney with acute tubular necrosis, strong ARHGDIB expression was seen in endothelial cells of interlobular arteries, endothelial cells of peritubular capillaries, and endothelial cells of glomerular capillaries. In addition,

FIGURE 1 Impact of cut-off for the presence of non-HLA antibodies against ARHGDIB on graft survival. A, In this figure the hypothesis is displayed that the presence of a non-HLA antibody is associated with graft failure. B, Using acquired data from non-HLA measurements, we determined the difference in graft survival between the non-HLA antibody negative and positive group for various cut-off values at 1, 5, and 10 years after transplantation in a univariate Kaplan-Meier analysis. Here, the results for directly coupled ARHGDIB are used as an example. The highest difference in graft survival between the ARHGDIB-positive and -negative group was achieved with a cut-off for signal-to-background ratio of 10 in combination with a cut-off for absolute MFI of 500. The graft survival difference for this cut-off between the ARHGDIB-positive and -negative group was 5.9%, 10.9%, and 13.1% at 1, 5, and 10 years after transplantation, respectively. C, Shown are the percentages of ARHGDIB-positive patients for each cut-off. For the selected cut-off there are 2.8% (134 of 4770) positive patients



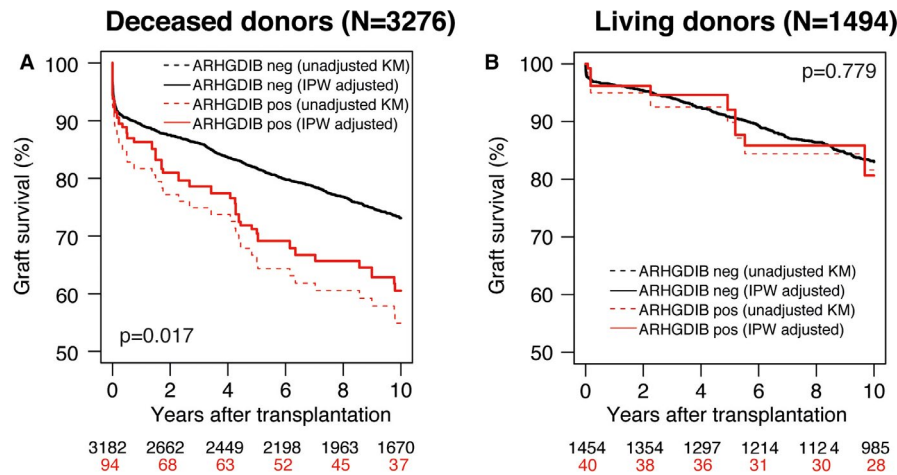


FIGURE 2 Graft survival according to the presence of pretransplant non-HLA antibodies in deceased donor and living-donor kidney transplantation. Inverse probability weighting (IPW) adjusted Kaplan-Meier estimate (AKME) for death-censored graft survival according to the presence of ARHGDIB in 3276 deceased (A) and 1494 living-donor (B) transplantations. AKME was adjusted for the following covariates: recipient age (quadratic) and donor age (quadratic), cold ischemia time (for donation after brain death and donation after cardiac death), time on dialysis in years (quadratic), induction therapy with IL-2 receptor blocker, and the presence of pretransplant donor-specific anti-HLA antibodies (DSA)

positive staining for ARHGDIB is also seen in some podocytes and lymphocytes (Figure 3B).

4 | DISCUSSION

In the present study, we determined the relation between graft failure and the presence of pretransplant non-HLA antibodies in the sera of 4770 kidney transplantations performed in The Netherlands between 1995 and 2006. The results indicate that pretransplant antibodies against ARHGDIB represent a risk factor for graft loss in deceased-donor transplantations.

The 14 non-HLA target proteins included in our assay were selected based on reported antibody specificity in kidney transplant patients or patients with end-stage renal disease, and their expression in the kidney. A number of these non-HLA antibodies have been examined in relation to kidney transplantation. Antibodies against AT1R were associated with rejection and graft survival according to results from several large-scale studies,^{5,20} although a recent large study ($n = 940$) did not confirm this association.⁴ Pretransplant anti-ETAR antibodies have been associated with higher serum creatinine values at 1 year posttransplantation and with more severe vascular rejection.²¹ Antibodies against LG3, a C-terminal fragment of perlecan, were associated with acute tubulointerstitial rejection and long-term renal allograft dysfunction.^{15,22} Antibodies against PLA2R, LPLUNC, APMAP, and PRKCZ were described previously in small case-series and have not been further evaluated.²³⁻²⁵ During the development of our non-HLA antibody assay we found that MFI results on a specific bead was comparable between singleplex and multiplex measurement, indicating no occurrence of compromise by multiplex analysis. In general, non-HLA antibodies frequently occur within the

healthy population and for many autoantibodies a serum is considered positive when exceeding a cut-off yielding a limited (<5%) frequency of positive results within the healthy population. This definition does not necessarily provide the most clinically relevant cut-off value with regard to prognosis. To this end, we chose to define a clinically relevant cut-off as it was previously described for HLA antibodies using a combination of STBR combined with a minimal MFI level.²⁶ Using individual cut-offs for each non-HLA antibody, optimally discriminating patients with long-term functioning grafts vs graft loss, we did not find associations between the presence of any of the abovementioned previously studied non-HLA antibodies and graft loss, function, or rejection. We did not analyze the autoantibody MFI levels as continuous variables, because in the field of anti-HLA antibodies it is well accepted that MFI is not an indication of antibody titer and is influenced by several factors such as (1) the antigen density, (2) the affinity of the antibody to the antigen, and (3) the amount/titer of the antibody (also dependent on the serum dilution used in the assay).

At present, no studies have been reported showing the association between anti-ARHGDIB antibodies and graft loss. Here, we found significant associations between graft loss and the pretransplant presence of antibodies against ARHGDIB. The effect of these antibodies is observed predominantly in patients transplanted with a deceased-donor kidney. This suggests an interaction between the presence of anti-ARHGDIB antibodies and ischemia reperfusion injury, which is less prominent in living-donor kidney transplantation.²⁷ ARHGDIB is widely expressed, including in the renal pelvis and glomeruli. Increased expression of ARHGDIB has been reported in several solid tumors and hematological malignancies.²⁸⁻³¹ Bilalic et al³² first described that dialysis patients can have autoreactive antibodies against ARHGDIB. Further analysis in kidney biopsies showed cytoplasmic expression in endothelial cells of interlobular

TABLE 2 Characteristics of ARHGDIB-positive and ARHGDIB-negative patients transplanted with a deceased-donor kidney

Characteristics	ARHGDIB negative (N = 3182)	ARHGDIB positive (N = 94)	P-value	Deceased-donor transplantations (N = 3276)
Patient				
Age at transplantation (y, mean \pm SD)	46.9 \pm 14.1	47.9 \pm 13.4	.462 ^a	46.1 \pm 14.1
Female sex - no. (%)	1286 (40.4)	39 (41.5)	.834 ^b	1325 (40.5)
PRA at time of transplantation (% , mean \pm SD)	6.8 \pm 18.7	9.2 \pm 25.1	.574 ^a	6.9 \pm 18.9
Highest PRA (% , mean \pm SD)	16.4 \pm 28.2	15.4 \pm 30.4	.300 ^a	16.4 \pm 28.2
Dialysis, n (%)			.455 ^b	
No	147 (4.6)	3 (3.0)		150 (4.6)
Yes – hemodialysis	1853 (58.2)	49 (52.1)		1902 (58.1)
Yes – peritoneal dialysis	1164 (36.6)	41 (43.6)		1205 (36.8)
Unknown	18 (0.6)	1 (1.1)		19 (0.6)
Time on dialysis (y, mean \pm SD)	3.4 \pm 2.6	3.4 \pm 2.2	.814 ^a	3.4 \pm 2.6
Donor				
Donor age (y, mean \pm SD)	42.7 \pm 16.0	46.0 \pm 14.6	.062 ^a	42.8 \pm 16.0
Donor female sex – no. (%)	1489 (46.8)	46 (48.9)	.681 ^b	1535 (46.9)
Cold-ischemia time (hours, mean \pm SD)	21.8 \pm 7.2	23.5 \pm 7.7	.043 ^a	21.8 \pm 7.2
Transplant				
Repeat transplantation – no. (%)	554 (17.4)	16 (17.0)	.922 ^b	570 (17.4)
Pretransplant DSA against HLA-A/B/DR/ DQ – no. (%)	423 (13.3)	10 (10.6)	.454 ^b	433 (13.2)
Induction therapy				
IL-2 receptor blocker – no. (%)	651 (20.5)	14 (14.9)	.186 ^b	665 (20.3)
T-cell depleting antibody ^c no. (%)	134 (4.2)	0 (0)	.042 ^b	134 (4.1)
Initial immunosuppression – no. (%)				
Steroids	3120 (98.1)	90 (95.7)	.117 ^b	3210 (98.0)
MMF/azathioprine	2377 (74.7)	63 (67.0)	.092 ^b	244 (74.4)
Cyclosporine/ tacrolimus	3000 (94.3)	88 (93.6)	.785 ^b	3088 (94.3)
Sirolimus	171 (5.4)	9 (9.6)	.078 ^b	180 (5.4)
Other	417 (13.1)	11 (11.7)	.691 ^b	428 (13.1)
Unknown	11 (0.4)	1 (1.1)	.256 ^b	12 (0.4)

DSA, Donor-specific anti-HLA antibodies; IL, interleukin; MMF, mycophenolate mofetil.

^aMann-Whitney *U* test for continuous variables.^bChi-square test for categorical variables.^cT cell-depleting antibody therapy: ALG, ATG, OKT3 monoclonal antibodies.

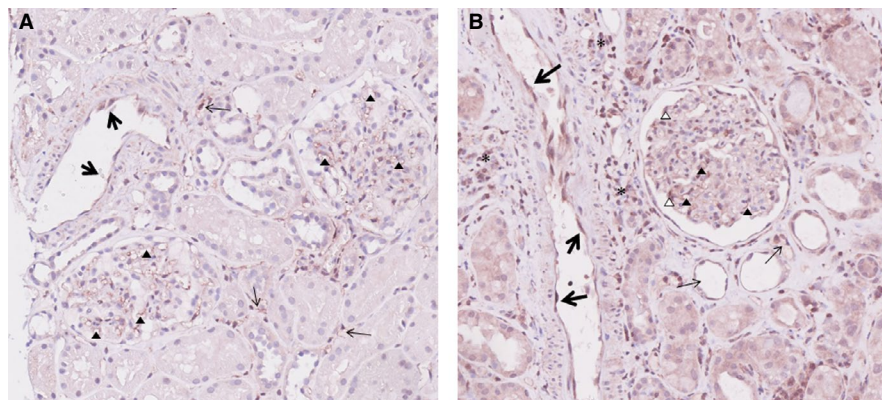


FIGURE 3 ARHGDI B expression in the kidney. A, In a transplanted kidney without histological abnormalities, weak ARHGDI B expression is seen in endothelial cells of interlobular arteries (large arrows), endothelial cells of peritubular capillaries (small arrows), and endothelial cells of glomerular capillaries (arrow heads). B, In a transplanted kidney with acute tubular necrosis, strong ARHGDI B expression is seen in endothelial cells of interlobular arteries (large arrows), endothelial cells of peritubular capillaries (small arrows), and endothelial cells of glomerular capillaries (solid arrow heads). In addition, positive staining for ARHGDI B is also seen in some podocytes (open arrow heads) and lymphocytes (asterisks)

arteries and peritubular capillaries, and in podocytes. We consider it possible that cellular damage caused by ischemia reperfusion results in accessibility of ARHGDI B in endothelial cells to circulating auto-antibodies. After binding, anti-ARHGDI B antibodies may initiate complement activation causing local inflammation, which may explain why a relation between their presence and graft loss was observed only after deceased-donor transplantation.

In our study, non-HLA proteins were coated directly or indirectly (via HaloTag) to the microspheres. This strategy was chosen because the coupling process may influence the conformation and accessibility of epitopes predominantly recognized on non-HLA beads.¹¹ Previously we found that the correlation between results obtained with our test and with commercial assays depended on the coupling method of the proteins. This demonstrates the necessity of complete transparency and detailed description of methods when comparing the effects of non-HLA antibodies in patient groups. Because there are no commercially available reference sera against the non-HLA antibodies we selected, we were not able to properly compare our Luminex assay to other commercially available ELISA or Luminex autoantibody assays. In the sera used in our multiplex assay, we measured non-HLA IgG antibodies. It is possible that some non-HLA antibodies relevant to prognosis are of another isotype and were not detected here. In some autoimmune diseases, autoantibodies are of other isotypes, such as IgM-RF or IgA-anti-tTG. However, most clinically relevant autoantibodies are of the IgG isotype. We also examined whether a reactivity pattern between non-HLA antibodies can be distinguished, for instance due to cross-reactivity, but we did not find significant associations between each of the 14 non-HLA antibodies investigated and there was no relationship with pretransplant DSA.

In a large cohort studied by Opelz, a 9% difference in 10-year graft survival was observed between recipients of HLA-identical sibling donors with or without panel-reactive HLA antibodies, which led to the conclusion that a high immunization grade against HLA may indicate an increased immunity against non-HLA.³³ In our study, the observed

adverse effects of non-HLA antibodies were independent of the presence of DSA as we adjusted for this covariable. Because ARHGDI B is considered to be a minor histocompatibility antigen, we examined the relation between antibody levels and potentially immunizing events, but found no link with repeat transplantation, female sex, pregnancies, or potentially confounding factors such as diabetes type 1, or several primary renal diseases. Therefore, mechanisms may be involved in the induction of non-HLA antibody formation other than the well-known sensitizing events stimulating HLA antibody production.

We were not able to include posttransplant samples (sera and/or biopsies) in our study, thereby limiting the use of results in pretransplant risk stratification. Future studies have to be performed to evaluate whether posttransplant monitoring of anti-ARHGDI B is useful. Another limitation of the study was that we did not have detailed clinical information, such as autoimmune diseases or hypertension, to potentially link the presence of anti-ARHGDI B antibodies to a clinical parameter. Due to lack of detailed rejection and histology information, we cannot directly link anti-ARHGDI B antibodies with the rejection phenotype.

In conclusion, our study demonstrates that pretransplant non-HLA antibodies against ARHGDI B are a significant risk factor in deceased-donor transplantation. These antibodies occur independently from DSA or other non-HLA antibodies investigated. It is currently unknown whether the presence of these antibodies is a biomarker, as is the case in many autoimmune diseases, or whether they play a role in the pathogenesis of graft loss. Although validation of our findings in independent cohorts is necessary, based on these results, we suggest that pretransplant risk assessment can be improved by measuring these antibodies in all patients awaiting deceased-donor transplantation.

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DISCLOSURE

The authors of this manuscript have no conflicts of interests to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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